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Measurement of urine indolylacroylglycine (IAG) is not useful in the diagnosis or dietary management of autism

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Lay Abstract

Increased urinary excretion of indolylacroylglycine (IAG) has been proposed as a useful biomarker for the diagnosis and dietary management of patients with autism spectrum disorder (ASD). It has been suggested that this may be due to abnormal metabolism of tryptophan and/or evidence of increased gut permeability to peptides, and may be a valuable biomarker of a process underlying autism viz. the hypothesis that ASD could be caused by increased gut absorption of neuroactive peptides, derived from gluten in wheat and casein in milk, harmful to the brain. This hypothesis has supported the use of gluten- and casein-free diets in those with ASD, and testing of urine for IAG as a prelude to advising dietary manipulation in individuals with ASD. However, there are conflicting reports of whether or not people with ASD have increased amounts of IAG in their urine, the fundamental finding on which the hypothesis above is built. One recent study found increased IAG in the urine of children with persistent gastrointestinal problems. The present study compares IAG in the urine in children with ASD, children with special needs, and in typically developing children and finds no difference among the groups. Neither was there any difference in those with and without current gastrointestinal problems. We found no evidence of increased IAG excretion or for any link to abnormal tryptophan metabolism and/or gut permeability in children with ASD. The data suggest that the measurement of urinary IAG has no value in the diagnosis of ASD or in the dietary management of patients with ASD.

Scientific abstract

Objective. To measure urine indolylacroylglycine (IAG) excretion using the IAG:creatinine ratio in children with autism spectrum disorder (ASD) compared with two groups of age matched controls, one with special needs but without ASD (SEN) and one typically developing (TD) and in subgroups with/without current gastrointestinal problems and ASD with and without regression.

Participants and methods. IAG:creatinine ratio was measured in the urine of 279 children aged 10-14 years: 129 children with ASD (28 with and 101 without regression), 62 SEN controls and 88 TD controls. The prevalence of gastro-intestinal symptoms (GIS) was recorded.

Results. No differences were found in the urine IAG:creatinine ratio among groups ASD, TD and SEN; nor in the ASD groups with/without regression, nor in those with/without GIS.

Conclusions. This study finds no evidence of increased urine IAG excretion in children with ASD, with or without GIS or with or without regression. Urinary IAG measurements in children with ASD offer no support for increased presence of neuroactive peptides proposed to result from increased gut permeability. We found measurement of urinary IAG to have no value in the diagnosis of autism or in the dietary management of children with ASD.

Key words: indoleacroylglycine (IAG), gastrointestinal, regression

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Introduction

In the search for metabolic abnormalities in autism, one group of investigators reported greatly increased concentrations of indolylacrylglycine (IAG) in the urine of participants with autism (Anderson et al., 2002) and subsequently suggested that IAG in urine may be a biomarker for autism (Bull et al., 2003). IAG is a metabolic product of the amino acid tryptophan and can be found in the urine of normal healthy individuals. Hypotheses of processes responsible for increased IAG excretion invoke altered tryptophan metabolism by abnormal gut flora promoting conversion of tryptophan to indolyl propionic acid, which is then absorbed and converted to IAG (Smith and MacFarlane, 1997); increased gut permeability; a failure of phosphorylation of tryptophan hydroxylase due to binding of opioid receptors by A-gliadin in those unable to metabolise gluten, giving rise to excess opioid peptides in the urine; or the use of organo-phosphates as pesticides.

Despite the absence of any formal scientific evidence, several groups considered the data provided a metabolic rationale for a gluten- and casein-free (GFCF) diet in autism and suggested dietary intervention for children with ASD found to have particular patterns of urinary findings (Whiteley et al., 1999; Shattock and Whitely, 2002; Knivsberg et al., 2002, and reported further in Alcorn et al., 2004). Subsequent research has been unable to confirm these earlier findings, although the GFCF diet continues to be widely used (Salomone et al., 2015), sometimes on the basis of prediction from urinary testing. A small study (Hunter et al., 2003) could not confirm the presence of neuroactive peptides in urine from children with autism and a subsequent large rigorously conducted study of urinary opioid peptides found no difference between control subjects and those with autism (Cass et al., 2008). Studies have failed to find any evidence of gut permeability problems in children with autism (Dalton et al., 2014) or increased absorption/excretion of potentially neuroactive peptides (Dettmer et al., 2007). Wright et al. (2005) analysed urine from matched population groups, with and without autism, expressing their results as IAG/ creatinine ratios, to control for urine dilution, and found no difference in IAG excretion. Wang et al. (2009)

also found no evidence to suggest increased IAG excretion in children aged 3-8 years with autism compared with typically developing siblings and a community control group. However, Wang et al. (2009) did find higher urinary IAG excretion and higher IAG:creatinine ratio in a subgroup of children with ASD who had persistent gastrointestinal symptoms (GIS) compared with no persistent GIS and both control groups, whether or not they had persistent GIS (elicited by the question ‘Does your child have any chronic/ongoing gastrointestinal issues i.e., bloating, diarrhoea, constipation, excessive flatulence, abdominal pain’?). The increased IAG:creatinine ratio was thus specific to children with ASD and GIS. Wang et al. (2009) suggested IAG may be a useful biomarker for ASD with associated GIS which could assist with the selection of targeted interventions to normalise GIS such as probiotics, prebiotics and/or antibiotics. They recommended further research to replicate their findings. Thus we investigated urine IAG:creatinine ratio in a population-derived sample of children with well-defined ASD with two control groups, same-age children with special educational needs but without autism (SEN) and typically developing children (TD). Subgroups defined were those with and without current GIS elicited by parental questionnaire and those with ASD with and without a history of regression elicited by parental interview as those with regression have particularly been hypothesised as having GIS (Valicenti-McDermott et al., 2008).

Methods

This study received ethical approval from the following Ethics Committees: South Thames (ref MREC 00/1/50) and Kent & Medway LREC (ref WK153/8/02).

Sample

The sampling methodology of the SNAP study (funded by the Wellcome Trust) has been described previously (Baird et al., 2006). In brief, within a total population cohort of 56,946 children born between July 1st 1990 and December 31st 1991, all with a current clinical diagnosis of ASD

(N=255) or considered ‘at risk’ by virtue of having a Statement of Special Educational Needs¹ (SEN; N=1,515) were screened using the Social Communication Questionnaire (SCQ) (Rutter et al., 2003). A stratified subsample (by coincidence also N=255) based on SCQ score received a comprehensive diagnostic assessment by trained researchers which included standardized clinical observation (Autism Diagnostic Observation Schedule – Generic (ADOS-G); Lord et al. 2000), parent interview assessments of autistic symptoms (Autism Diagnostic Interview-Revised (ADI-R); Lord et al. 1994), language and intelligence (IQ), psychiatric comorbidities (Simonoff et al., 2008) and a medical examination. All information including ADI-R and ADOS-G was used to derive a clinical consensus diagnosis of ASD (childhood autism and other ASDs; Baird et al. 2006) based on ICD-10 research criteria. Regression was defined as in the ADI –R as loss of words or babble with regression of social and play skills (Baird et al., 2008). Cases not meeting criteria for a diagnosis of ASD were categorized as SEN; these children had special educational needs and a variety of other diagnoses including ADHD, cerebral palsy, language disorders and intellectual disability. Parental report of bowel problems was collected using a questionnaire administered by researcher interview with the parents/carers of the ASD and SEN participants. The 20-item GIS questionnaire asked about current (last 3 months) and past (prior to the last 3 months) symptoms (Chandler et al., 2013). Cases were categorized as having current GIS if any of the following 4 symptoms were reported: current diarrhoea (3 or more times a day), current abdominal pain (severe enough to interfere with activity), current vomiting (at least once per day or more than 5 times in a week) or current constipation.

A further 98 typically developing (TD) controls (no special educational needs) were recruited from two mainstream schools within the same geographical area. Parents were mailed information about the study, a consent form, SCQ and GIS questionnaire. The latter was completed by post rather

¹A Statement of Special Educational Needs is a legal document issued by the local educational authority when children require significant additional support in school due to any learning and/or behavioural problems.

than direct interview. An SCQ score of at least 15 was used to screen out possible cases of ASD (Berument et al., 1999).

Urine samples, without preservative, were obtained in participants who were able to provide one on the day of assessment. The samples were stored at -80°C until analysis.

Analytical methods

Urine IAG was measured using liquid chromatography stable isotope dilution positive ion electrospray tandem mass spectrometry (MSMS). IAG and stable isotope labelled IAG (d3-IAG) were synthesised commercially and aqueous stock 1mM solutions stored in aliquots at -80°C. Aqueous working standards, 0.1 to 100µmol/l, were prepared from an aliquot of stock standard on the day of analysis. Working stable isotope internal standard was prepared by diluting stock d3-IAG (1mM) 1:400 with the chromatography solvent (acetonitrile:water (1:1) with 0.025% formic acid). Urine samples for analysis were thawed, mixed, and centrifuged at 20,000 rpm for 2min. 25µl of standard, quality control, or sample were diluted with 225µl of working internal standard directly into a 96 deep well (2ml) polypropylene plate. Samples (2µl) were injected automatically (CTC autosampler, Presearch Ltd) into a solvent flow (200µl/min) and chromatographed isocratically on a Symmetry C8 3.5µm 2.1 x 50mm column with guard column. Positive ion (5250V) electrospray MSMS was performed in multiple reaction monitoring mode on an API4000 triple quadrupole mass spectrometer (Applied Biosystems, Warrington) tuned to optimise the m/z 244.9 to 170.1 and 247.9 to 170.1 signals. Results were calculated in Analyst 1.4. Assay performance: sensitivity 0.1 µmol/L, linear to at least 100µmol/l (measured sample concentrations ranged from 2-100µmol/L), recovery 100%, within batch imprecision 2.8%, and between batch imprecision 3.7%. Urine creatinine was measured using liquid chromatography stable isotope dilution positive ion electrospray MSMS as previously described (Dalton et al., 2014).

Data Analysis

Data analysis was performed in Stata11 (StataCorp). The concentrations of urine analytes, including IAG, are seldom informative because of the wide range of urine dilution, dependent on fluid intake. Analyte excretion rates, requiring complete bladder emptying and accurate timing, are not practical either in children or the majority of adults. Consequently, urine analytes are conventionally expressed as a ratio to creatinine, which is excreted at a relatively constant rate and it is this ratio that we report here. Individuals with ASD were compared with SEN and TD controls. Due to the skewed nature of the IAG:creatinine data, non-parametric statistics were used to test for group differences in IAG:creatinine ratio. The ASD group was further sub-divided into ASD with and without a history of regression. Spearman's rank correlations were used to test for any correlation between current GIS scores (range 0-4) and IAG:creatinine ratio within each diagnostic group. ANCOVA was used to further examine the effects of group (TD control/SEN control/ASD) and GIS (absence/presence of current GIS) on IAG:creatinine ratio. For this, the raw IAG:creatinine data were naturalised using logarithmic transformation due to the skewed nature of the data; the natural log of the IAG:creatinine ratio was then entered as the dependent variable, with GIS (presence/absence of current symptoms) and group (TD control/SEN control/ASD) as the independent variables, and age as a covariate due to the positive correlation between age and creatinine levels ($\rho(278)=.13, p=.03$). (A trend towards a decline in urinary IAG:creatinine ratio with age in ASD children was found by Wright et al. (2005) and by Wang et al. (2009)).

Results

IAG:creatinine ratios were measured in 129 children with ASD, 62 children with special educational needs (SEN) but no ASD and 87 typically developing children, a total of 278 children. The ASD group was sub-categorised into ASD with ($n=28$) and without ($n=101$) regression. The bowel questionnaire was completed on 252 of the 278 who produced urine samples (116 with ASD, 57 SEN, and 79 TD controls).

Group characteristics are summarized in table 1.

Table 1 about here

No significant group differences were found in IAG:creatinine ratio, either between the ASD, SEN and TD groups (median IAG:creatinine ratio (95% C.I.) = 3.42 (2.85 – 3.85), 3.84 (3.38-4.22) and 3.41 (3.01-3.96) respectively, Kruskal Wallis, $p = .63$), or between the ASD groups with and without regression (median IAG:creatinine ratio (95% C.I.) = 3.42 (2.99-4.09) and 3.27 (2.49-4.83) respectively, Wilcoxin rank-sum, $p = .90$). Figures 1a and 1b present the IAG:creatinine ratios by group. High IAG:creatinine ratio outliers were observed in all of the groups and probably reflect increased dietary tryptophan intakes (Marklova, 1999) .

Figure 1 about here

The rate of GIS was highest among the ASD group (see Table 1). However, no significant correlation was found between GIS score (0-3) and IAG:creatinine for the total sample, or for the ASD group (either as a whole or when ASD cases with and without regression were tested separately (Spearman rank correlations, all $p > .14$)). A low correlation was found between GIS score and IAG:creatinine within the SEN group only ($\rho(57) = .29, p = .03$). Figure 2 presents the IAG:creatinine ratios for each group, with and without GIS symptoms.

Figure 2 about here

To further examine the effect of group and presence/absence of GIS on IAG:creatinine ratios, a 2x2 ANOVA was performed on the natural log of IAG:creatinine ratio, with age entered as a covariate. No significant main effects or interaction were found ($F(6, 251) = .89, p = .50$).

Discussion

In this population-derived sample, there is no evidence of a clinically significant difference in IAG:creatinine ratio among children with ASD, SEN without ASD and typically developing children, providing confirmation of the conclusions drawn by Wright et al. (2005) and Wang et al. (2009). Furthermore, there was also no significant difference in IAG:creatinine ratio between those with and without current GIS in typically developing children and those with ASD, in contrast to Wang et al. (2009) who found that urinary IAG and the IAG:creatinine ratio were increased in a sub-set of children with autism with ongoing gastrointestinal symptoms. Similar definitions of GIS appear to have been used in both studies, but while the current study used a series of detailed questions to ascertain the presence of gastrointestinal symptoms, Wang et al. asked one single yes/no question and it is not clear how “unsure” responses were treated. Participants in the Wang et al. (2009) study were younger (mean age 6 years 10 months) than in the present study (mean age 11 years 6 months) which also may have accounted for the difference in prevalence of GI symptoms. We found a low correlation between GIS score and IAG:creatinine within the SEN group, who represent a small number and a mixed diagnostic group.

Strengths of this study are a large, well-characterised sample, 129 with ASD of whom 27 had concurrent GIS. All laboratory analysis was blind to participant status. The analysis we used was similar to that used by both Wright et al. (2005) and Wang et al. (2009). Earlier studies were typically unblinded and based on small, volunteer samples (e.g., Bull et al, 2003; Anderson et al., 2002).

Conclusions.

We conclude that urine IAG measurements have no role in the diagnosis of autism and provide no evidence of an underlying abnormality of tryptophan metabolism in the development of ASD or of

an increased presence of neuroactive peptides proposed to result from increased gut permeability. The results provide no scientific evidence to support the hypotheses underlying GFCF diets for autism symptoms or offering urinary IAG testing to parents of children with autism as a prelude to or justification for dietary intervention.

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Conflict of interest disclosure:

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Table 1. Group characteristics

	TD controls	SEN	ASD	ASD without regression	ASD with regression
	N=87	N=62	N=129	N=101	N=28
Age - yrs (mean (SD))	12.2 (0.3) ^{††}	12.6 (0.9) ^{**}	11.5 (0.8) ^{**, ††}	11.6 (0.8)	11.5 (0.9)
Range	11.6-12.8	10.1-14.0	10.0-13.8	10.0-13.5	10.3-13.8
IQ (mean (SD))	n/a	78.9 (20.3)	73.6 (22.2)	76.0 (22.3) [*]	65.0 (20.2) [*]
Range	n/a	31-131	19-136	28-136	19-105
SCQ total (mean (SD))	4.37 (3.58) ^{**, ††}	9.1 (7.8) ^{**, ΔΔ}	23.9 (6.9) ^{, ††, ΔΔ}	23.2 (7.2) [*]	26.6 (4.9) [*]
Range	0-14	0-31	2-37	2-36	15-37
ADI-R total (mean (SD))	n/a	12.1 (8.6) ^{**}	43.8 (11.8) ^{**}	41.9 (12.2) ^{††}	50.5 (7.0) ^{††}
Range		0-34	0-62	0-62	36-61
ADOS-G total (mean (SD))	n/a	4.02 (3.05) ^{**}	12.7 (6.39) ^{**}	11.5 (6.0) ^{††}	17.1 (6.1) ^{††}
Range		0-11	0-28	0-28	4-27
% Male (n)	60.9 (n=53) ^{*, ††}	85.5 (n=54) [,] , [*]	88.4 (n=114) ^{††}	87.1 (n=88)	92.9 (n=26)
% with current GIS	11.4 [*] (9/79)	14.0 (8/57)	23.3 [*] (27/116)	26.9 (25/93)	8.7 (2/23)

*t-test, Fisher's exact, $p < .05$ **, ††, ΔΔ t-test, Chi-square, $p < .001$

Figure 1a. Boxplots of IAG:creatinine ratio by group, indicating median, lower and upper quartiles and outliers

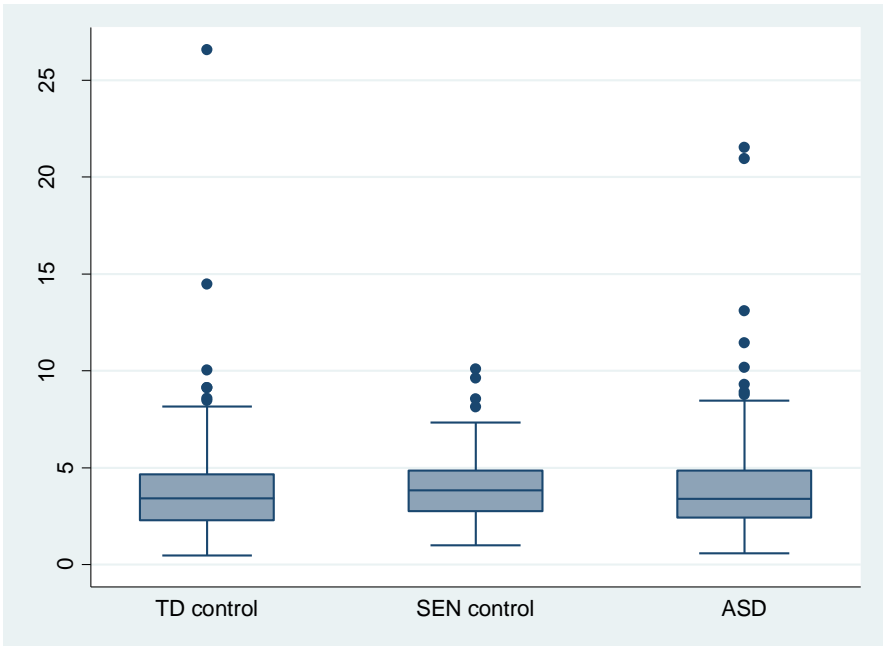


Figure 1b. Boxplots of IAG:creatinine ratio for ASD groups with and without regression, indicating median, lower and upper quartiles and outliers

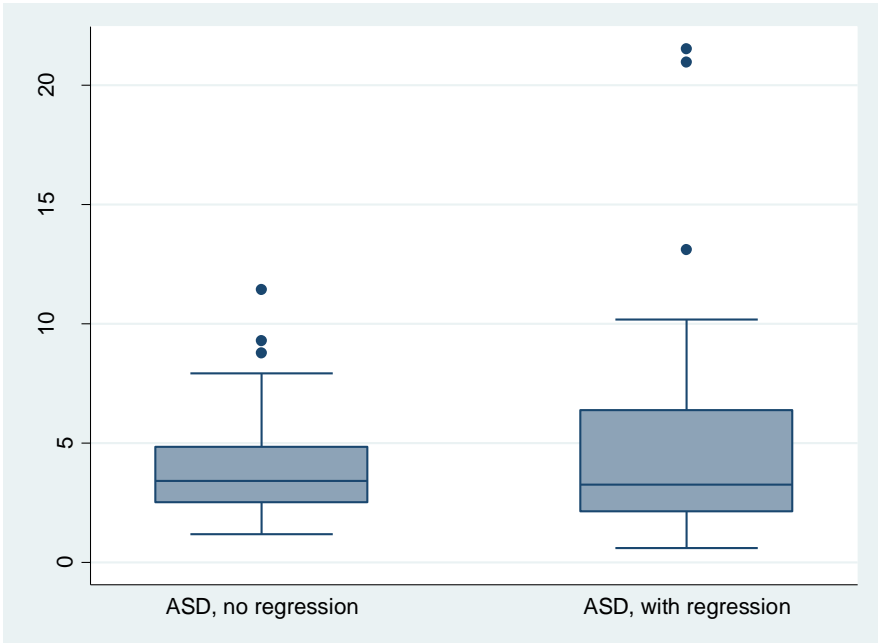
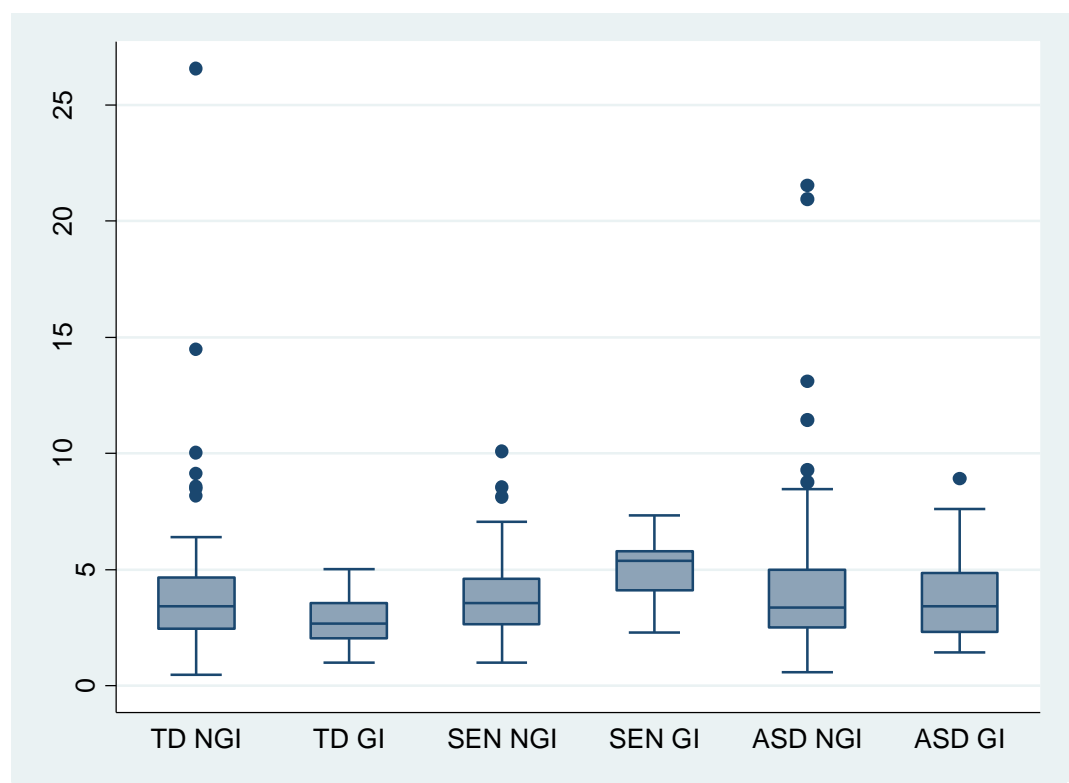


Figure 2. Boxplots of IAG:creatinine ratio for the diagnostic groups, with and without current GIS



NGI = No gastrointestinal symptoms present

GI = Gastrointestinal symptoms present